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RESEARCH ARTICLE

Biomarkers in cerebrospinal fluid for amyotrophic lateral sclerosis phenotypes

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Abstract

Objective: Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease involving both upper and lower motor neurons. The motor phenotypes of ALS are highly clinically heterogeneous, and the underlying mechanisms are poorly understood. **Methods**: A comparative proteomic analysis was performed in the cerebrospinal fluid (CSF) of bulbar-onset (BO) and spinal-onset (SO) ALS patients and controls (n = 14). Five biomarker candidates were selected from a differentially regulated protein pool, and further validation was performed in a larger independent cohort (n = 92) using enzyme-linked immunosorbent assay (ELISA). **Results**: A total of 1732 CSF proteins were identified, and 78 differentially expressed proteins were found among BO-ALS patients, SO-ALS patients, and controls. Five promising biomarker candidates were selected for further validation, and lipopolysaccharide-binding protein (LBP) and HLA class II histocompatibility antigen, DR alpha chain (HLA-DRA) were validated. CSF LBP levels were increased in ALS patients compared with controls and higher in BO-ALS versus SO-ALS. The increased CSF LBP levels were correlated with the revised ALS Functional Scale (ALSFRS-R) score. CSF HLA-DRA levels were specifically elevated in BO-ALS patients, and there was no significant difference between SO-ALS patients and controls. Increased HLA-DRA expression was correlated with decreased survival. **Interpretation**: Our data shows that elevated CSF LBP is a good biomarker for ALS and correlates with clinical severity, and increased HLA-DRA is a specific biomarker for BO-ALS and may predict short survival. It also suggests that the microglial pathway and HLA-II-related adaptive immunity may be differentially involved in ALS phenotypes and may be new therapeutic targets for ALS.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder that gradually affects upper motor neurons and lower motor neurons.[1] The motor phenotypes of ALS are well recognized to be highly clinically heterogeneous in terms of onset location, relative mix of upper motor neuron and lower motor neuron involvement, and progression rate.[2] According to the body region of onset, ALS has been divided into three phenotypes: spinal onset (SO), bulbar onset (BO), and respiratory onset.[2] The symptoms of SO-ALS start with asymmetric, painless weakness in a limb, and clinical examination usually reveals atrophy and weakness of muscles, fasciculations, hyperreflexia, and hypertonia. In BO-ALS, the weakness starts in bulbar muscles, and dysarthria, dysphagia, and tongue fasciculations occur. In respiratory-onset ALS, patients present with orthopnea or dyspnea and mild or even absent spinal or bulbar signs. It is widely accepted that patients with BO-ALS have a worse prognosis than patients with SO-ALS, with significantly shorter survival.[3] Respiratory-onset ALS has the worst prognosis among the

three phenotypes, with a mean survival of 1.4 years and no long-term survival.[4] Although there are numerous active themes in the study of the pathogenesis of ALS, little is known about the mechanisms contributing to phenotypic variety. Whether these clinical phenotypes of ALS correspond to different pathological changes in the central nervous system remains unclear.

ALS is pathologically characterized by degeneration and loss of motor neurons and reactive gliosis, and several mechanisms have been implicated in the pathophysiology. However, their relative contributions to pathogenesis are poorly understood. The search for biomarkers can pave the way to a deeper understanding of its multifactorial pathophysiological processes and provide new targets for disease-modifying treatment strategies. The most promising ALS biomarker candidates are neurofilaments in the cerebrospinal fluid (CSF)[5,6] and blood.[7,8] Neurofilaments are neuron-specific cytoskeletal components comprising the axonal cytoskeleton and maintaining neuronal caliber. Elevated phosphorylated neurofilament heavy chain (pNFH) and neurofilament light chain (NFL) concentrations have been found in the CSF and blood of ALS patients compared with controls. However, altered neurofilament levels are also observed in other neurological diseases, including multiple sclerosis, Parkinson's disease, and multiple system atrophy,[9,10] suggesting that neurofilament is an unspecific marker of axonal damage/degeneration rather than a disease-specific marker.[11] TDP-43 is another promising ALS biomarker candidate because it accumulates in the nerve cells of ALS patients and may play a critical role in its pathogenesis.[12] Previous studies have attempted to evaluate disease-specific TDP-43 levels in biofluids. However, the results from the quantification of TDP-43 isoforms in the CSF of ALS patients have been contradictory.[12] Neuroinflammation is also a hot topic in ALS biomarker studies, and alterations in the levels of cytokines, chemokines, and complement proteins have been shown in previous studies.[11] The increased use of unbiased tools, including proteomics, is advancing biomarker discovery. Proteomics enables biomarker discovery by identifying disease-associated protein alterations.[13,14] Recent proteomic studies have reported differential expression of biomarker candidates including ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1), chitotriosidase-1 (CHIT1), microtubule-associated protein 2 (MAP2), and glycoprotein nonmetastatic melanoma protein B (GPNMB).[14,15] These proteins are linked to inflammatory processes and axonal health. However, there are few comparative analyses of biomarkers among different ALS phenotypes.

This study was planned to assess differentially expressed proteins in the CSF of BO-ALS patients, SO-ALS patients, and controls using tandem mass tag (TMT) labeling combined with multidimensional solid phase/liquid chromatography tandem mass spectrometry (LC–MS/MS). Enzyme-linked immunosorbent assay (ELISA) was used for further validation. This study aimed to discover novel biomarkers differentiating ALS phenotypes and provide a better understanding of the mechanisms of ALS heterogeneity.

Materials and Methods

Participants and sample collection

This study was approved by the Ethical Review Board at the Xiangya Hospital of Central South University (No. 202103201). All participants provided informed consent and underwent evaluation, comprising medical history collection, physical and neurological examinations, laboratory tests, and neuropsychological assessment. All methods were performed in conformity with the approved guidelines.

This study included 55 ALS patients and 51 sex- and age-matched controls (who were admitted to a neurological inpatient clinic but without a final diagnosis of degenerative or inflammatory nervous system disease, as shown in Table S1). ALS patients were diagnosed according to the revised El Escorial criteria by two senior neurologists.[16] The inclusion criteria for ALS patients were as follows: (1) age between 18 and 80 years old; (2) typical ALS presentation with both upper motor neuron and lower motor neuron involvement; (3) drug naivety; and (4) complete laboratory results indicating that the complete blood count and serum electrolyte, blood urea nitrogen, creatinine, glucose, vitamin B12 and thyroid-stimulating hormone levels were within normal limits. The exclusion criteria for ALS patients were (1) atypical ALS including pseudopolyneuritic ALS, flail arm syndrome, flail leg syndrome, progressive muscular atrophy, and primary lateral sclerosis; (2) patients with a family history of ALS or frontotemporal dementia, or known to carry a genetic mutation linked to ALS or frontotemporal dementia; (3) patients with other neurological diseases; and (4) patients who received riluzole or other anti-inflammatory or antioxidant drugs within 3 months prior to the study. The revised ALS functional rating scale (ALSFRS-R) was used to assess disease severity.[17] Based on the initial symptoms, the ALS group was further classified into SO-ALS ($n = 39$) and BO-ALS ($n = 16$) groups. The disease progression rate was defined as the difference between the maximum ALSFRS-R and the ALSFRS-R score at the date of CSF sampling divided by the disease duration at the date that the ALSFRS-R score was collected.[17] A disease progression rate lower than 0.43 points/month, corresponding to the 25th percentile, was

defined as slow progression; a disease progression rate between 0.43 and 1.4 points/month was defined as intermediate progression; and a disease progression rate higher than 1.4 points/month, corresponding to the 75th percentile, was defined as fast progression. Survival was defined as the time difference between the date of lumbar puncture (LP) and the date of death. All deaths occurred due to ALS rather than any other causes.

For the discovery set, four BO-ALS patients, five SO-ALS patients, and five controls were included in the comparative proteomic analysis. For validation, an independent cohort with 12 BO-ALS patients, 34 SO-ALS patients, and 46 controls was included in the ELISA test. The experimental process is shown in Figure 1. All CSF samples were obtained by LP under nonfasting conditions, aliquoted and stored at −80°C within 1 h.

Sample preparation, TMT labeling, and fractionation

For protein extraction, a Pierce™ Albumin/IgG Removal Kit was used to remove the highly abundant proteins albumin and IgG in each sample, and 150 μL of solution was obtained from each group. The protein concentration was measured with a 2-D Quant kit (GE Healthcare) according to the manufacturer's instructions. Then, the products were reduced with 10 mM dithiothreitol (DTT, purchased from Sigma) for 1 h at 37°C and alkylated with 20 mM iodoacetamide (IAA, purchased from Sigma) for 45 min at room temperature in darkness. Afterwards, protein samples were diluted by adding 100 mM TEAB to urea concentrations less than 2 M. Finally, trypsin was added with a trypsin-to-substrate mass ratio of 1:50 for the first digestion overnight and a 1:100 trypsin-to-protein mass ratio for the second 4-hour digestion. Approximately 100 μg protein for each sample was digested with trypsin for the following experiments. Peptides were then desalted by a Strata X C18 SPE column (Phenomenex) and vacuum dried. The desalted peptides were reconstituted in 0.5 M TEAB and labeled according to the manufacturer's protocol for the 6-plex TMT kit (Thermo). The peptide mixtures were incubated for 2 h at room temperature and pooled, desalted, and dried by vacuum centrifugation. Samples were mixed and fractionated by high-pH reverse-phase high-performance liquid chromatography (HPLC) using an Agilent 300Extend C18

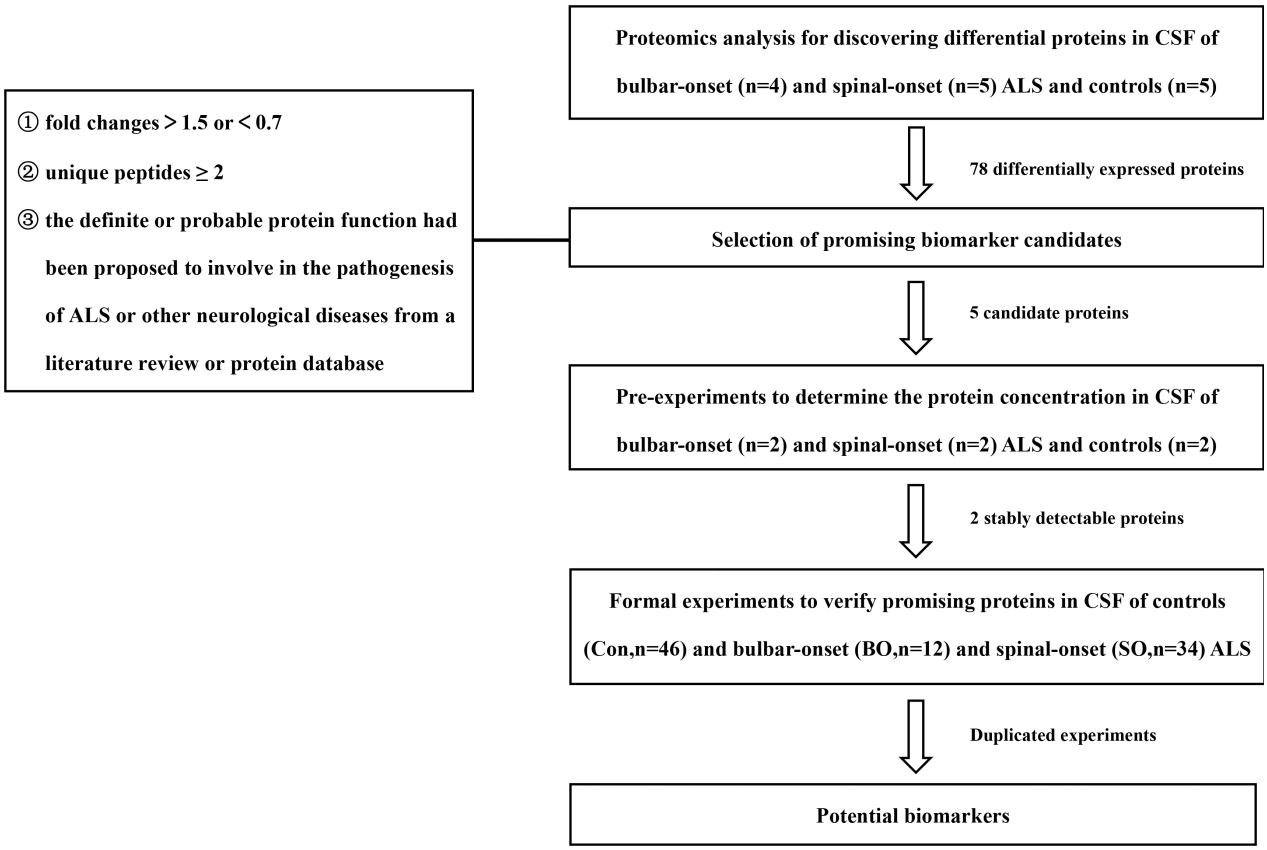

Figure 1. Flow chart showing the experimental process.

column (5 μm particles, 4.6 mm ID, 250 mm length). The peptides were initially separated with a serial gradient of acetonitrile with concentrations ranging from 2% to 60% in 10 mM ammonium bicarbonate (pH 10) over 80 min. The collected 80 fractions were further combined into 18 fractions, which were dried by vacuum centrifugation for LC–MS/MS analysis.

LC–MS/MS analysis

Peptides were dissolved in 0.1% formic acid (FA) and directly loaded onto a reversed-phase precolumn (Acclaim PepMap 100, Thermo). Peptide separation was performed via a reversed-phase analytical column (Acclaim PepMap RSLC, Thermo). The gradient was established as an increase from 6% to 23% solvent B, which was a mixture of 0.1% FA and 98% acetonitrile, over 26 min, then 23% to 35% in 8 min, and finally elevated to 80% in 3 min, and the concentration was maintained at 80% for another 3 min, all at a constant flow rate of 400 nl/min on an EASY-nlC 1000 ultra-performance liquid chromatography (UPLC) system. The separated peptides were subjected to a nanospray ionization (NSI) source followed by MS/MS analysis in Q Exactive TM plus (Thermo) coupled online to the UPLC. The electrospray voltage used was 2.0 kV. Intact peptides were detected in the Orbitrap at a resolution of 70,000, while ion fragments were detected in the Orbitrap at a resolution of 17,500. A total of 50,000 ions were accumulated for the generation of MS/MS spectra. For MS scans, the scan range was 350–1800 (m/z), and the fixed first mass was set as 100 (m/z). Peptides were selected for MS/MS using a normalized collision energy (NCE) setting of 32. A data-dependent procedure that alternated between one MS scan and 20 MS/MS scans was applied for the top 20 precursor ions above a threshold ion count of 10,000 in the MS survey scan with 30.0 s dynamic exclusion.

Proteomics database search

The resulting LC–MS/MS data were processed via MaxQuant with the integrated Andromeda search engine (v.1.5.2.8). Tandem mass spectra were searched in the Swiss-Prot database (*Homo sapiens*) concatenated with a reverse decoy database. Trypsin/P was specified as a cleavage enzyme allowing up to two missing cleavages and five modifications per peptide. The mass error was set to 10 ppm for precursor ions and 0.02 Da for a fragment, and the minimum peptide length was set at 7. Carbamidomethylation on Cys was specified as a fixed modification, and oxidation on Met and acetylation on the protein N-terminus were specified as variable modifications. False discovery rate (FDR) thresholds for protein, peptide, and modification sites were specified at 1%. Gene Ontology (GO) terms were derived from the UniProt-GOA database (http://www.ebi.ac.uk/GOA/) for all proteins identified, including cellular components, molecular functions, and biological processes. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/) was used to annotate differential protein pathways.

ELISA

The level of candidate protein in CSF was measured by commercial sandwich ELISA kits according to the manufacturer's instructions (listed in Table S2). Generally, 96-well plates were coated overnight at room temperature with protein antibodies, followed by incubation of the standards and CSF with the corresponding biotinylated detection antibodies for 1 h at 37°C. After three washes, horseradish peroxidase (HRP)-conjugated reagents were added to the plate and incubated for 40 min at 37°C, followed by thorough washing steps. Then, 3,3′,5,5′-tetramethylbenzidine (TMB) was applied, and after stopping the reaction, the absorbance was measured immediately at 450 nm on a Tecan Genios multifunctional microplate reader. The sigmoidal standard was evaluated with a nonlinear four-parameter fit using curve expert 1.30 software, and sample amounts were measured using the fitted standard curve. All tests were performed in duplicate.

Statistical analysis

The proteomic analysis was accomplished in the R (version 3.2.2) statistical environment. Statistical analysis of patient characteristics and ELISA validation analyses were performed with SPSS (version 26.0). Graphs were generated using GraphPad Prism (version 9.3.0). In the analysis of the discovery cohort, sex distribution was analyzed by Fisher's exact test. Groups were compared by the Mann–Whitney U test (two groups) or Kruskal–Wallis test and Dunn's post hoc test (>2 groups). A two-tailed Fisher's exact test was performed to analyze the enrichment of the differentially expressed protein among all identified proteins in GO term enrichment analysis and KEGG pathway enrichment analysis, and correction for multiple hypothesis testing was carried out using standard FDR control methods. In the analysis of the validation cohort, Pearson chi-square and Fisher's exact tests were used to describe the sex distribution between ALS and controls and between BO-ALS and SO-ALS patients, respectively. Other baseline demographics and clinical characteristics were compared by the Mann–Whitney U test or *t* test. Correlations between parameters were calculated by the

Spearman rank correlation coefficient (r_s). Generalized linear models were used to compare the concentration of candidate proteins between ALS and controls, covarying with age at LP and sex. Analysis of covariance (ANCOVA) was applied to differentiate CSF protein levels between BO-ALS and SO-ALS patients, with age at LP, sex, and disease duration as covariates. To explore factors affecting the expression of proteins within ALS patients, simple linear regression followed by multiple linear regression was performed. Receiver operating characteristic (ROC) curves were generated to estimate the area under the curve (AUC), and optimal cutoffs were estimated via the highest Youden's index. At optimal cutoffs, sensitivity and specificity were defined to estimate the performances of the candidate protein to discriminate between ALS patients and controls or within ALS subtypes. Survival was assessed with Kaplan–Meier curves, and the log-rank test was utilized to assess the differences between the survival curves. Univariable and multivariable analyses with the Cox proportional hazards model were used to estimate the simultaneous effects of prognostic factors on survival. The significance for all tests was set at $p < 0.05$.

Results

Demographic characteristics of the study cohorts

Four BO-ALS patients, five SO-ALS patients, and five controls were recruited for discovery analysis, and 12 BO-ALS patients, 34 SO-ALS patients, and 46 controls were recruited for the validation study. In the discovery cohort, there were no significant differences in age at LP and sex among the three groups. Within the ALS groups, the onset age of BO-ALS patients was significantly older than that of SO-ALS patients. There were no significant differences in disease duration or ALSFRS-R score between BO-ALS and SO-ALS patients. In the validation cohort, there was no significant difference in age at LP or sex between ALS patients and controls. Within the ALS groups, the onset age and age at LP of BO-ALS patients were significantly higher than those of SO-ALS patients. Sex, disease duration, ALSFRS-R score, and progression rate were not significantly different between the BO-ALS and SO-ALS groups. Demographic characteristics are presented in Table 1.

Table 1. Demographic and clinical characteristics of patients with ALS and controls.

	Proteomic analysis cohort			
	BO	SO	Con	*p*
n	4	5	5	NA
Sex (male/female)	3/1	4/1	3/2	1.000[a]/1.000[b]/1.000[c]
Age at LP (years)	64.25 ± 8.02	52.00 ± 6.44	42.60 ± 14.74	0.050[a]/0.142[b]/0.050[c]
Onset age (years)	63.25 ± 8.02	51.20 ± 6.26	NA	0.049
Disease duration (months)	10.50 ± 3.00	6.20 ± 2.95	NA	0.132
ALSFRS-R score (points)	38.25 ± 2.36	39.60 ± 2.30	NA	0.319

	ELISA validation cohort					
	ALS	Con	*p*[d]	BO	SO	*p*[e]
n	46	46	NA	12	34	NA
Sex (male/female)	33/13	24/22	0.053	8/4	25/9	0.704
Age at LP (years)	54.57 ± 9.67	52.63 ± 13.26	0.426	62.17 ± 7.18	51.88 ± 9.05	<0.001
Onset age (years)	53.48 ± 9.45	NA	NA	61.08 ± 6.95	50.79 ± 8.79	<0.001
Disease duration (months)	13.15 ± 14.30	NA	NA	12.92 ± 16.08	13.24 ± 13.89	0.948
ALSFRS-R score (points)	39.22 ± 6.08	NA	NA	40.92 ± 3.78	38.62 ± 6.66	0.265
Disease progression rate (points/months)	1.60 ± 2.66	NA	NA	2.63 ± 4.68	1.24 ± 1.36	0.330

Most of data presented as mean ± SD. NA, not available. $p < 0.05$ was considered statistically significant.
ALS, amyotrophic lateral sclerosis; ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-revised; BO, bulbar-onset ALS; Con, controls; ELISA, enzyme-linked immunosorbent assays; LP, lumbar puncture; n, number; SO, spinal-onset ALS.
[a]BO versus Con.
[b]SO versus Con.
[c]BO versus SO.
[d]ALS versus Con.
[e]BO versus SO.

Discovery of CSF proteome analysis

The CSF proteomes of BO-ALS patients, SO-ALS patients and controls were analyzed by TMT-LC–MS/MS. In total, 1732 proteins with at least one unique peptide were identified (see Table S3). Proteins detected in ≥3 samples in at least one group were used for quantitative comparisons (1530 proteins). A total of 78 proteins were differentially expressed between groups, including 43 upregulated and 6 downregulated proteins in BO-ALS patients versus controls, 11 upregulated and 19 downregulated proteins in SO-ALS patients versus controls, and 12 upregulated proteins in BO-ALS patients versus SO-ALS patients (fold changes >1.2 or <0.83, FDR <0.05; Fig. 2 and Tables S4–S6). Several differential proteins overlapped between groups. To further explore the potential functional alterations in ALS patients, we performed GO enrichment and KEGG pathway enrichment of differentially expressed proteins. Enrichment analysis of differential proteins between ALS patients and controls revealed an overrepresentation of proteins involved in the activation and regulation of the immune response, hsa04610 complement and coagulation cascades, and the hsa04142 lysosome pathway, which was reported in our previous study.[18] Regarding the differentially expressed proteins between BO-ALS and SO-ALS patients, GO enrichment analysis showed that they mainly comprised collagen trimers and were enriched in the molecular function of protein heterodimerization activity. They were also involved in regulation of the protein activation cascade, humoral immune response, protein maturation, defense response, positive regulation of the immune effector process and immune response, cartilage development, skeletal system development, and tissue homeostasis (Fig. 3A–C). In KEGG pathway enrichment analysis, the differentially expressed proteins between BO-ALS and SO-ALS patients were primarily involved in the hsa05322 systemic lupus erythematosus and hsa05168 Herpes simplex virus 1 infection pathways (Fig. 3D; Figs S1 and S2).

Among the 78 differentially expressed proteins, neurofilament medium polypeptide (NFM) and UCHL1, which had been widely reported to be upregulated in ALS patients[14,15,19] and had no significant differences between BO-ALS and SO-ALS patients in our discovery test as well as previous studies,[14,20] were not further validated here. Furthermore, the five most promising changed proteins [lipopolysaccharide-binding protein (LBP), protein FAM19A5 (FAM19A5), transmembrane gamma-carboxyglutamic acid protein 1 (PRRG1), apolipoprotein L1 (APOL1), and human leukocyte antigen (HLA) class II histocompatibility antigen, DR alpha chain (HLA-DRA)] were selected as biomarker candidates for further validation based on the following criteria: (1) fold changes >1.5 or <0.7; (2) unique peptides ≥2; and (3) have been proposed to be involved in the pathogenesis of ALS or other neurological disorders from a literature review or protein database (www.uniprot.org) (Table 2 and Table S7).

Validation of selected promising biomarker candidates

Before the formal validation experiment, preliminary tests of five candidate proteins were performed in CSF using commercial ELISA kits (Cusabio and Cloud-Clone Corp). LBP and HLA-DRA levels were found to be stably detected in each sample. Hence, further validation tests of LBP and HLA-DRA were performed in the CSF of 12 BO-ALS patients, 34 SO-ALS patients, and 46 controls.

Comparison of protein levels between ALS patients and controls

In all cases, generalized linear analysis was performed with the disease-control group, covarying with age at LP and sex. The CSF concentration of LBP was significantly higher in ALS patients than in controls ($\chi^2 = 4.25$, $p = 0.039$; Fig 4A). There were no significant effects of age at LP ($p = 0.559$) or sex ($p = 0.596$) on LBP levels. The CSF concentration of HLA-DRA was not significantly different between ALS patients and controls ($p = 0.711$; Fig. 4C). The level of HLA-DRA was affected by age at LP ($p = 0.013$) but not sex ($p = 0.238$).

Correlation between age and protein level

In the control group, a positive correlation between age at LP and CSF HLA-DRA level was observed ($r_s = 0.47$, $p < 0.001$; Fig 4E). This correlation was not found in the ALS group ($p = 0.141$). No correlation between age at LP and CSF LBP levels was found in either the control ($p = 0.925$) or ALS group ($p = 0.229$).

Protein level within ALS subtypes

Considering that the onset age and age at LP of BO-ALS patients were significantly older than those of SO-ALS patients, ANCOVA was used to determine the difference in the protein levels between patients with BO and SO subtypes, covarying with age, disease duration, and sex. Patients with BO-ALS had higher LBP and HLA-DRA levels than patients with SO-ALS ($F = 11.59$, $p = 0.002$ for LBP, Fig. 4B; $F = 7.42$, $p = 0.009$ for HLA-DRA, Fig 4D). To identify factors affecting candidate protein concentrations in ALS patients, simple linear regression followed by multiple linear regression was performed with sex, onset age, age at LP, site of onset, disease duration,

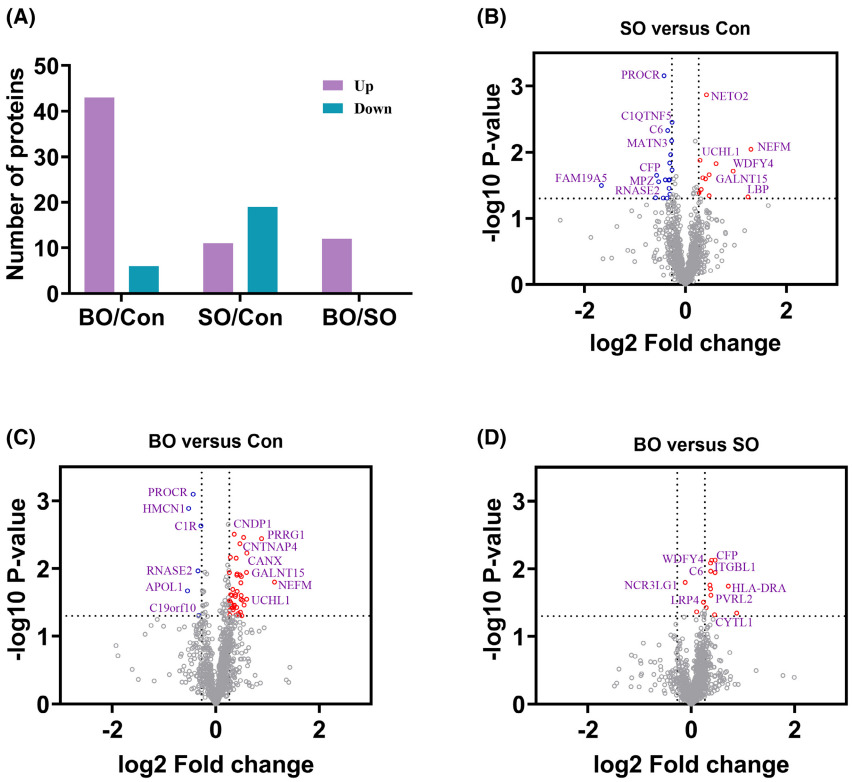

Figure 2. Significant proteomic changes in the CSF of ALS patients and controls. (A) Overall description of significantly differentially expressed proteins with fold changes above 1.2 or below 0.83 and FDR <0.05. Proteins with fold changes above 1.2 were considered upregulated, whereas those with fold changes below 0.83 were considered downregulated. Seventy-eight differentially expressed proteins were observed. (B–D) Volcano plots comparing protein levels in the CSF of SO-ALS patients versus controls (B), BO-ALS patients versus controls (C), and BO-ALS patients versus SO-ALS patients (D). Upregulated proteins are highlighted in red, and downregulated are highlighted in blue. ALS, amyotrophic lateral sclerosis; BO-ALS, bulbar-onset ALS; Con, controls; CSF, cerebrospinal fluid; Down, downregulation; FDR, false discovery rate; SO-ALS, spinal-onset ALS; Up, upregulation.

ALSFRS-R score, and progression rate as covariates. In simple linear regression analysis, the CSF level of LBP was correlated with the site of onset ($p = 0.012$) and ALSFRS-R score ($p = 0.024$), while HLA-DRA concentration was correlated with the site of onset ($p < 0.001$), onset age ($p = 0.027$), and age at LP ($p = 0.038$). Multiple linear regression showed that the site of onset ($p = 0.021$) and ALSFRS-R score ($p = 0.043$) were correlated with the CSF concentration of LBP, while the CSF concentration of HLA-DRA was only affected by the site of onset ($p = 0.009$). The results of linear regression are listed in Tables 3 and 4.

Discrimination assessment

ROC analyses showed that a cutoff of LBP levels of 174.74 ng/mL provided the optimal discrimination between ALS patients and controls at a sensitivity of 91.1% (95% CI, 59.53%–74.01%) and a specificity of 86.50% (95% CI, 63.92%–76.89%), with an AUC of 0.88 (95% CI, 0.79–0.97, Fig. 5A). Within the ALS group, a cutoff of LBP levels of 214.22 ng/mL and a cutoff of HLA-DRA levels of 500.42 pg/mL were appropriate for differentiation between BO-ALS and SO-ALS (sensitivity of 72.73% and specificity of 79.41%, AUC of 0.74 for LBP, Fig. 5B; sensitivity of 63.64% and specificity of 94.12%, AUC of 0.76 for HLA-DRA, Fig 5C). Combining LBP and HLA-DRA levels increased the AUC value to 0.77 with a sensitivity of 72.73% and specificity of 82.35% for differentiating BO-ALS from SO-ALS patients (Fig. 5D).

Survival analyses

Based on Kaplan–Meier survival curves, the survival of slow, intermediate and fast progressors significantly differed (log-rank test: $\chi^2 = 6.53$, $p = 0.038$, Fig 6A). The survival of patients based on the different tertiles of CSF

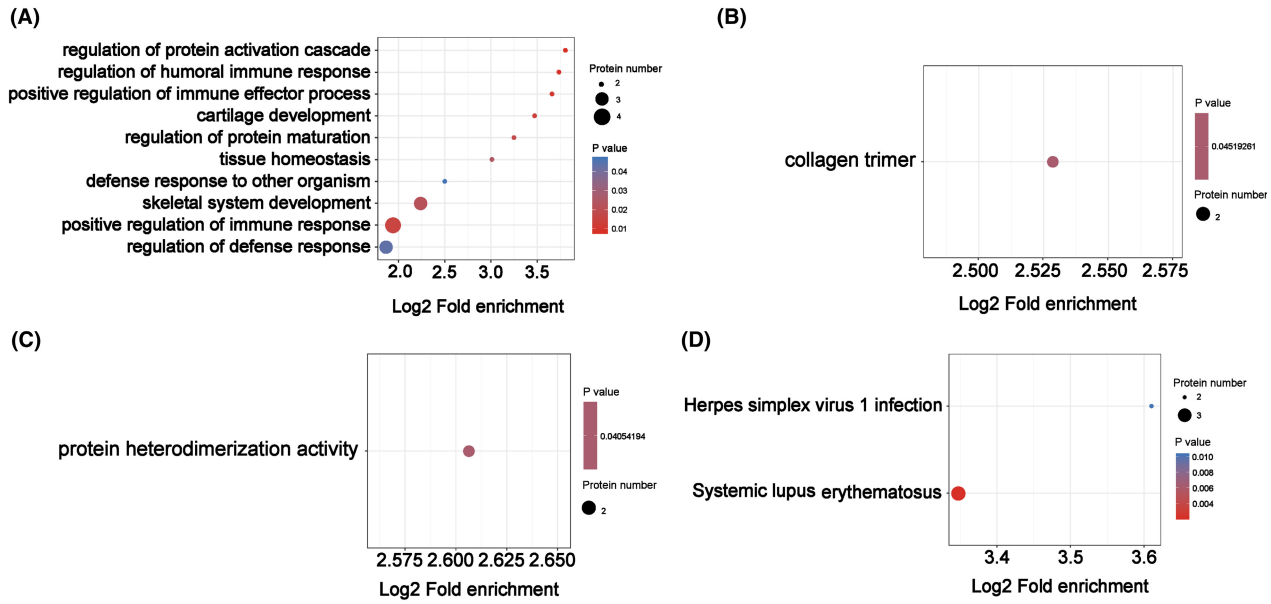

Figure 3. Functional enrichment analysis of differentially expressed proteins between bulbar- and spinal-onset ALS patients. (A–C) Scatter plots of enriched gene ontology (GO) terms, covering biological process (A), cellular component (B), and molecular function (C). (D) Scatter plot of enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. For each category, a two-tailed Fisher's exact test was employed to test the enrichment of the differentially expressed protein against all identified proteins. Log2-fold enrichment measures the number of differential proteins in the specific GO term or KEGG pathway. The larger the log2-fold enrichment, the higher the degree of enrichment.

Table 2. Promising biomarker candidates for validation.

Protein description	Gene name	Fold changes	Unique peptides	Comparison group	Regulated Type	FDR*
Lipopolysaccharide-binding protein	LBP	2.36	4	SO vs. Con	Up	0.047
Protein FAM19A5	FAM19A5	0.32	2	SO vs. Con	Down	0.032
Transmembrane gamma-carboxyglutamic acid protein 1	PRRG1	1.85	2	BO vs. Con	Up	0.004
Apolipoprotein L1	APOL1	0.69	10	BO vs. Con	Down	0.021
HLA class II histocompatibility antigen, DR alpha chain	HLA-DRA	1.64	2	BO vs. SO	Up	0.018

BO, bulbar-onset amyotrophic lateral sclerosis (ALS); Con, controls; FDR, false discovery rate; SO, spinal-onset ALS.
*SO versus Con/BO versus Con, FDR <0.05 was considered statistically significant.

level of HLA-DRA was also significantly different (log-rank test: $\chi^2 = 7.87$, $p = 0.020$, Fig 6B). Although the survival of BO-ALS patients was shorter than that of SO-ALS patients, there was unexpectedly no statistical significance (21.00 versus 32.20 months, $p = 0.205$). The univariate Cox regression of sex, onset age, site of onset, ALSFRS-R points, and tertiles CSF level of LBP or HLA-DRA revealed that ALSFRS-R points ($p = 0.041$) and CSF level of HLA-DRA ($p = 0.009$) were predictors of survival (Table 5). The variables with $p < 0.1$ and those identified as prognostic factors in a previous study[21] were added as survival covariables to the multivariate Cox regression, including CSF levels of LBP and HLA-DRA, onset age, and ALSFRS-R score. The concentration of CSF HLA-DRA ($p = 0.042$) was an independent predictor of survival (Fig. 6C and Table 6).

Discussion

In the present study, we investigated proteomic changes in CSF from ALS patients with BO and SO. We identified 1732 proteins and found 78 differentially expressed proteins among BO-ALS patients, SO-ALS patients and controls. Enrichment analysis indicated that the immune response and lysosome pathway were severely affected in ALS patients, and immune-inflammatory pathways were

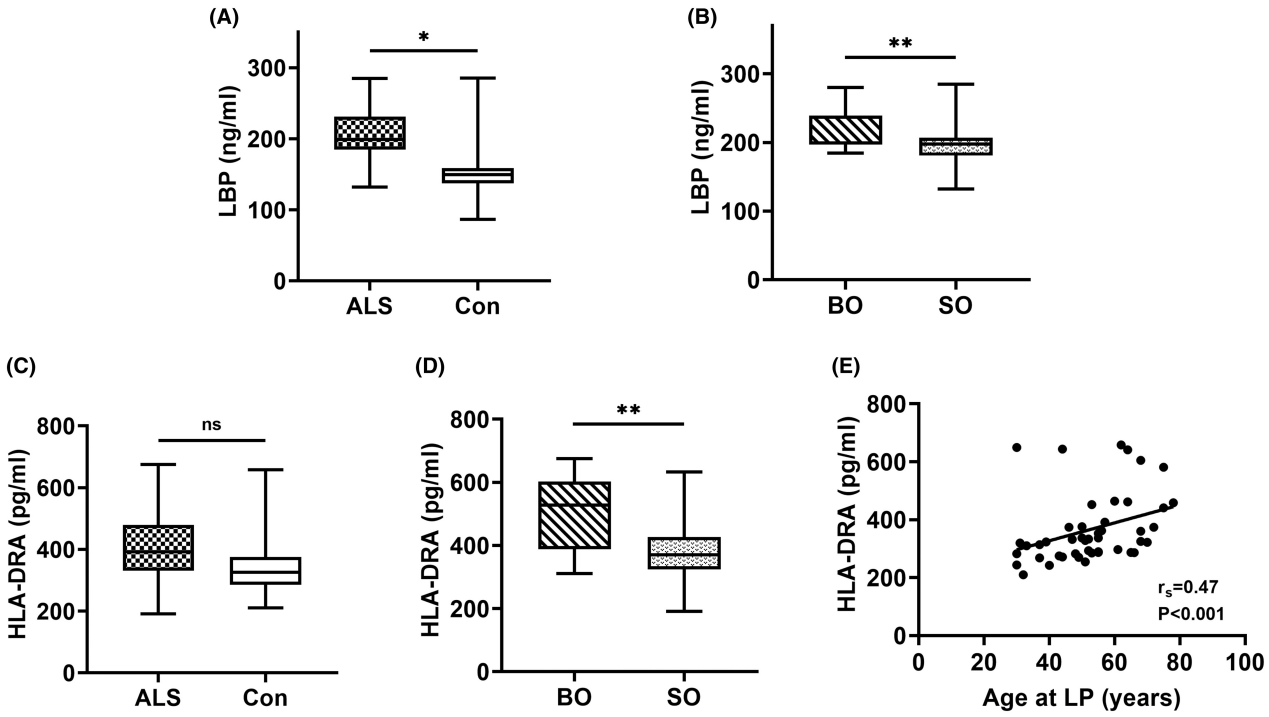

Figure 4. Comparison of LBP and HLA-DRA levels and correlation with age in the validation cohort. (A–D) Comparison of LBP and HLA-DRA expression in CSF between different groups. Generalized linear analysis with age at LP and sex as covariates was performed to evaluate differences between ALS patients and controls, and ANCOVA was used to determine the protein level between patients with BO and SO subtypes, with age, disease duration, and sex as covariates. The CSF concentration of LBP was significantly higher in ALS patients than in controls (A), while the CSF concentration of HLA-DRA was not significantly different between ALS patients and controls (C). Within ALS patients, the expression of LBP (B) and HLA-DRA (D) was significantly higher in BO-ALS patients than in SO-ALS patients. (E) Spearman rank correlation analysis showed a positive correlation between age at LP and CSF HLA-DRA level in controls. ALS, amyotrophic lateral sclerosis; BO-ALS, bulbar-onset ALS; CSF, cerebrospinal fluid; HLA-DRA, HLA class II histocompatibility antigen, DR alpha chain; LBP, lipopolysaccharide-binding protein; ns, no significance; SO-ALS, spinal-onset ALS. p value >0.05; *p value <0.05; **p value <0.01.

more often involved in the BO subtype. We further identified that CSF LBP was a novel biomarker discriminating ALS from controls and correlated with disease burden, while CSF HLA-DRA was a specific biomarker for BO-ALS and predicting survival. To our best knowledge, this is the first study to report alterations in LBP and HLA-DRA levels in the CSF of patients with different ALS phenotypes. Our findings suggest that neuroimmune-inflammatory pathways may be differentially involved in ALS phenotypes.

LBP is mainly produced in hepatocytes and released into the blood during the acute phase response, playing a pivotal role in the innate immune response. Circulating LBP seems to have a concentration-dependent immunologic function, and patients with high levels of LBP are prone to prolonged inflammation.[22] Higher peripheral LBP concentrations were observed in ALS patients than in controls in two recent studies,[23,24] and increased serum LBP levels were positively correlated with ALS burden and disease progression.[23] There have been no reports on the expression of LBP in the central nervous system of ALS patients. CSF is a good source for investigating central nervous disorders. In the current study, we discovered higher CSF LBP levels in ALS patients than in controls and higher levels in patients with BO-ALS than in patients with SO-ALS. Moreover, the elevated CSF LBP level was correlated with disease burden. This is the first study of the differential expression of LBP in the CSF of ALS patients and provides direct evidence that central LBP-related pathways are probably involved in ALS.

As an acute-phase protein, peripheral LBP levels are affected by widespread systemic inflammation. The changes in peripheral LBP levels in patients with neurodegenerative disorders were partially attributed to gut/oral dysbiosis. Whether peripheral immune alterations and inflammation directly reflect central changes remain under debate. The increased LBP levels observed in CSF in this study could have been due to infiltration resulting from the excessive levels of peripheral LBP through the disrupted blood–brain barrier or even overexpression in

Table 3. Simple linear regression of factors in predicting LBP and HLA-DRA concentration in CSF of ALS patients.

	LBP				HLA-DRA			
	B	Beta	95%CI	*p*	*B*	Beta	95%CI	*p*
Sex								
Female	Ref							
Male	6.13	0.09	−15.82-28.07	0.577	26.95	0.11	−48.66-102.57	0.476
Age at LP (years)	−0.40	−0.12	−1.44-0.63	0.437	3.62	0.31	0.22–7.03	0.038*
Onset age (years)	−0.37	−0.11	−1.42-0.69	0.489	3.93	0.33	0.47–7.40	0.027*
Disease duration (months)	−0.37	−0.16	−1.06-0.33	0.290	−1.08	−0.14	−3.48-1.32	0.369
ALSFRS-R score (points)	1.80	0.34	0.25–3.36	0.024*	1.10	0.06	−4.58-6.78	0.698
Disease progression rate (points/months)								
Low (<0.43)	Ref							
Intermediate	−12.61	−0.19	−36.95-11.74	0.302	8.70	0.04	−74.19-91.60	0.833
Fast (>1.40)	−12.64	−0.16	−41.85-16.58	0.388	−12.43	−0.05	−112.82-87.96	0.804
Site of onset								
Spinal	Ref							
Bulbar	28.14	0.37	6.58–49.70	0.012*	124.37	0.485	56.14-192.60	<0.001*

ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-revised; CI, confidence interval; HLA-DRA, HLA class II histocompatibility antigen, DR alpha chain; LBP, lipopolysaccharide-binding protein; LP, lumbar puncture; Ref, reference.
*Significance for $p < 0.05$.

situ. Abnormal LBP in the central nervous system could further bind to CD14, and the complex interacts with TLR4,[25,26] which promotes the neurotoxic phenotype of microglia and finally induces motor neuron death. Notably, the CSF LBP level was significantly correlated with the onset region and disease burden in our study. Onset location-based differential dysbiosis has been previously reported in ALS patients, with significant gut dysbiosis in SO-ALS patients and remarkable oral dysbiosis in BO-ALS patients. This phenomenon could be partially explained by the close anatomic connection between the gut and lower limbs[27] and the direct connection between the oral cavity and head/neck. Increased gut/oral dysbiosis is strongly associated with increased LBP blood levels and disease severity. Combined with these data, we speculate that increased gut/oral dysbiosis drives microbial translocation to the blood, producing blood LBP, which further infiltrates the central nervous system through a disrupted blood–brain barrier and activates the neurotoxic phenotype of microglia. Compared to gut dysbiosis, oral dysbiosis triggers more microbial translocation and higher LBP expression,[24] which injures the vessels/nerves supplying head and neck muscles[28] and leads to BO-ALS.

Table 4. Multiple linear regression of factors in predicting LBP and HLA-DRA concentration in CSF of ALS patients.

	B	Beta	95%CI	*p*
LBP				
ALSFRS-R score (points)	1.54	0.29	0.05–3.03	0.043*
Site of onset				
Spinal	Ref			
Bulbar	24.89	0.33	3.87–45.91	0.021*
HLA-DRA				
Age at LP (years)	−9.33	−0.79	−34.15–15.48	0.452
Onset age (years)	10.96	0.91	−14.61–36.54	0.392
Site of onset				
Spinal	Ref			
Bulbar	107.53	0.42	28.61–186.45	0.009*

*Significance for $p < 0.05$.
ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-revised; CI, confidence interval; HLA-DRA, HLA class II histocompatibility antigen, DR alpha chain; LBP, lipopolysaccharide-binding protein; LP, lumbar puncture; Ref, reference.

Elevated HLA-DRA levels were found in the CSF of BO-ALS patients in this study. HLA, also called the major histocompatibility complex (MHC), is the most complex genetic polymorphism system discovered thus far. HLA-II is encoded by three classical Class II genes (*HLA-DP, -DQ, -DR*) and comprises two noncovalently associated polypeptide chains, alpha and beta chains. It plays a vital role in the immune system by presenting peptides to the antigen receptor of helper T cells and regulating the differentiation of T cells.[29,30] Only cells expressing HLA-DR are able to induce T cells to respond, and this limited expression efficiently controls T-cell activation. In the central nervous system, HLA-II is expressed mostly by microglia, and low HLA-II expression is detected in astrocytes and endothelial cells.[31] Previous investigations have demonstrated that the expression of HLA-DR is increased with age in human brain tissue.[32,33] In this study, we

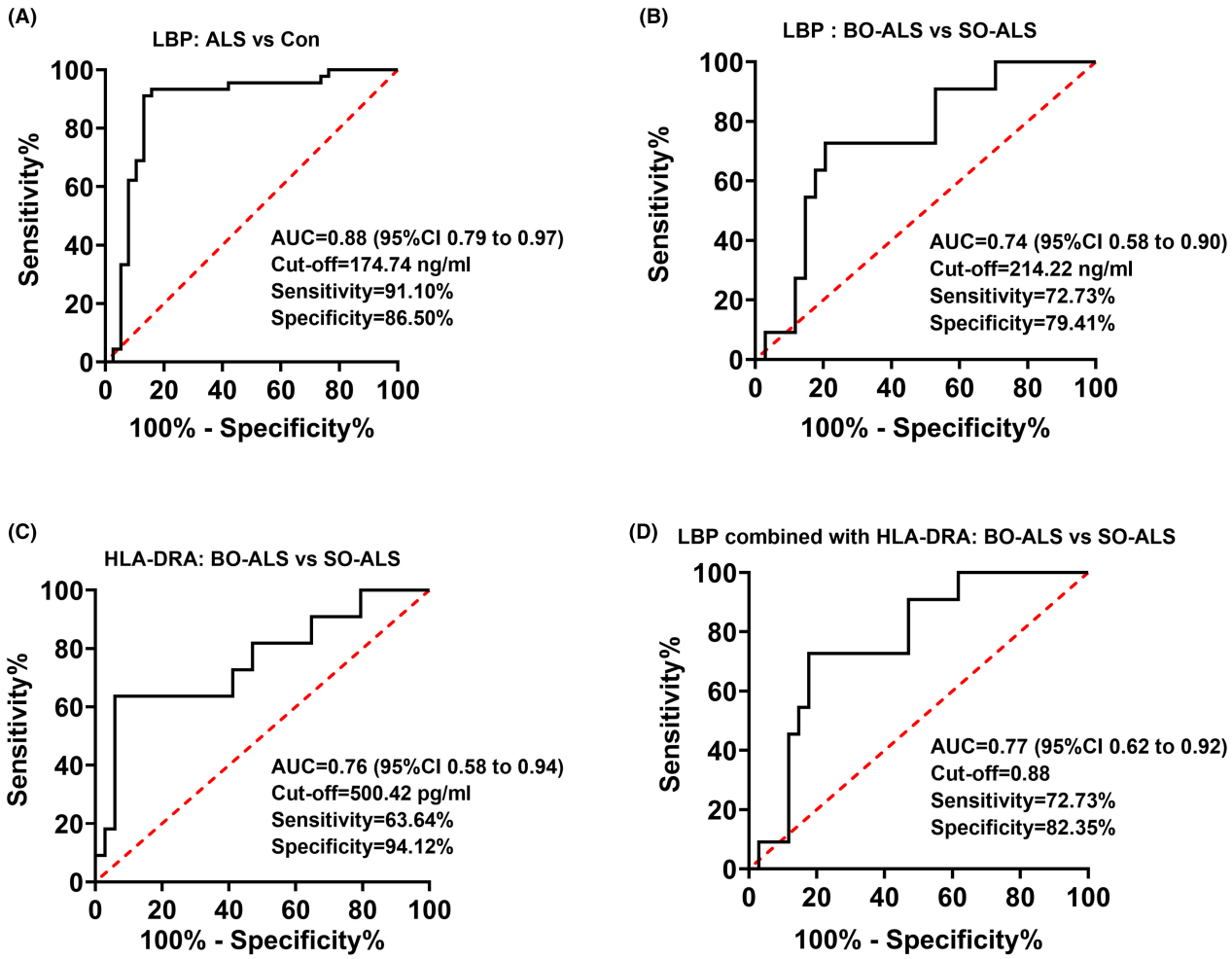

Figure 5. ROC analysis of LBP and HLA-DRA levels in CSF as ALS biomarkers. ROC curves of CSF LBP levels for discriminating ALS patients from controls (A), and discriminating BO-ALS from SO-ALS patients (B). ROC curves of CSF HLA-DRA levels for discriminating BO-ALS from SO-ALS (C). ROC curves of the combination of LBP and HLA-DRA levels in CSF discriminating BO-ALS from SO-ALS patients (D). ALS, amyotrophic lateral sclerosis; AUC, area under the curve; BO-ALS, bulbar-onset ALS; CSF, cerebrospinal fluid; HLA-DRA, HLA class II histocompatibility antigen, DR alpha chain; LBP, lipopolysaccharide-binding protein; ROC, receiver operating characteristics; SO-ALS, spinal-onset ALS.

identified the existence of HLA-DRA in human CSF and found a positive correlation between the CSF HLA-DRA concentration and age in controls, which supports the hypothesis of "inflamm-aging".[34] Moreover, the effects of disease onset location overwhelmed those of age, affecting the CSF concentration of HLA-DRA most in the ALS group. Markedly increased expression of HLA-DR has been observed in microglia of the motor cortex and spinal cord in ALS patients.[35,36] Microglia are the only resident parenchymal immune cells in the central nervous system and are usually described in two states: resting and activated. During a steady state, these cells are constantly surveying their environment, and upon injury, microglia migrate to the damaged areas and produce cytokines and neurotrophic factors to mitigate damage, participating in the neurotoxic or neuroprotective response, respectively.[37] In addition, activated microglia expressing HLA-DR can present antigens, thus inducing adaptive immune responses in neurodegeneration. Activated microglia may shift from a neuroprotective phenotype to a neurotoxic phenotype during disease progression.[38] Interestingly, we found that the CSF HLA-DRA levels were specifically increased in BO-ALS patients, and no significant difference in the CSF concentration of HLA-DRA was observed between SO-ALS patients and controls. In addition, increased HLA-DRA levels were correlated with decreased survival. These data indicate that the HLA-DRA-related microglial and T-cell activation pathways may be specifically involved in BO-ALS and contribute to worse outcomes.

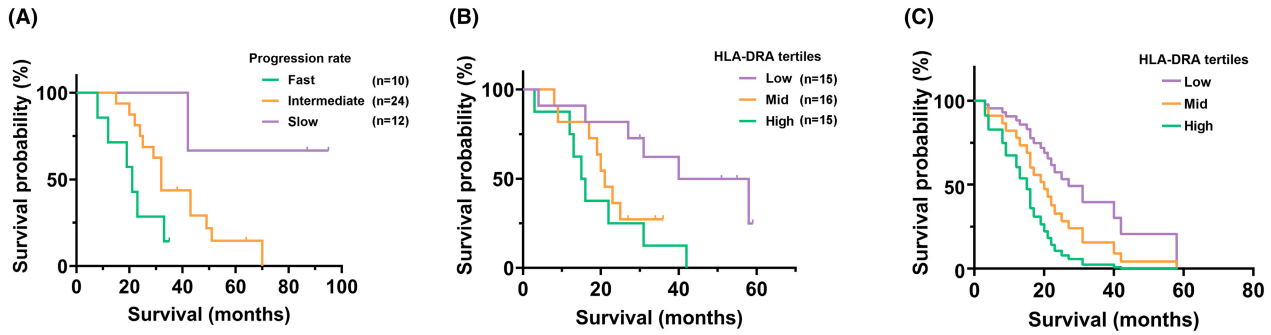

Figure 6. Survival analyses in the validation cohort. Disease progression was stratified into slow, intermediate and fast progression based on the changing rate of ALSFRS-R score during disease duration, and CSF levels of HLA-DRA were stratified into low, mid, and high tertiles. Kaplan–Meier curves were created for different progression rates (A) and tertiles of the CSF level of HLA-DRA (B). The CSF level of LBP, HLA-DRA, onset age, and ALSFRS-R points were entered in multivariate Cox regression models (C). ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-revised; CSF, cerebrospinal fluid; HLA-DRA, HLA class II histocompatibility antigen, DR alpha chain; LBP, lipopolysaccharide-binding protein.

Table 5. Univariate Cox proportional-hazards regression.

	B	χ^2	HR (95%CI)	*p*
Sex				
Female	Ref			
Male	0.78	2.55	2.19 (0.84–5.72)	0.110
ALSFRS-R score (points)	−0.06	4.20	0.95 (0.90–1.00)	0.041*
Site of onset				
Spinal	Ref			
Bulbar	0.62	1.54	1.86 (0.70–4.93)	0.215
Onset age (years)	0.04	2.83	1.04 (0.99–1.09)	0.092
LBP (ng/mL; tertiles)				
Low (<191.41)	Ref			
Mid	−0.07	0.01	0.94 (0.31–2.81)	0.906
High (>215.72)	0.24	0.22	1.27 (0.46–3.49)	0.642
HLA-DRA (pg/mL; tertiles)				
Low (<348.44)	Ref			
Mid	1.08	3.27	2.95 (0.91–9.52)	0.071
High (>438.67)	1.51	6.78	4.51 (1.45–14.01)	0.009*

*Significance for $p < 0.05$.
ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-revised; CI, confidence interval; HLA-DRA, HLA class II histocompatibility antigen, DR alpha chain; HR, hazard rate; LBP, lipopolysaccharide-binding protein; LP, lumbar puncture; Ref, reference.

Table 6. Multivariate Cox proportional-hazards regression.

	B	χ^2	HR (95%CI)	*p*
Onset age (years)	0.254	1.29	1.29 (0.83–2.00)	0.255
Age at LP (years)	−0.22	1.05	0.80 (0.52–1.22)	0.305
ALSFRS-R score (points)	−0.05	3.14	0.95 (0.90–1.00)	0.076
HLA-DRA (pg/mL; tertile)				
Low (<348.44)	Ref			
Mid	0.82	1.64	2.26 (0.65–7.87)	0.200
High (>438.67)	1.38	4.13	3.98 (1.05–1.22)	0.042*

*Significance for $p < 0.05$.
CI, confidence interval; HLA-DRA, HLA class II histocompatibility antigen, DR alpha chain; HR, hazard rate; LP, lumbar puncture; Ref, reference.

There are several limitations in this study. First, we did not assess proteins in peripheral blood. Increased LBP and HLA-DR levels in the peripheral blood of ALS patients have been reported in previous studies. It is unknown whether our findings of upregulated LBP and HLA-DRA in the CSF of ALS occurred in parallel with their changes in peripheral blood. Further studies to evaluate the relationship between peripheral and central LBP and HLA-DRA levels would benefit the determination of the effects of peripheral immunity in ALS. Second, the size of the BO-ALS group was much smaller than that of the SO-ALS group due to the low incidence. The imbalance of sample sizes between subgroups may cause bias, which could have led to the discrepant survival results in the different ALS subtypes between our study and previous reports. Further multicenter studies with larger sample sizes are needed to verify our findings.

Overall, our study presents an in-depth investigation of CSF proteins and indicates that LBP and HLA-DRA are potential biomarkers for ALS differentiation and disease prognosis. Additionally, these two proteins are promising candidates contributing to mechanisms underlying phenotypic variability. Along with previous reports, our findings indicate that activated microglial pathways and HLA-II-related adaptive immunity are differentially involved in ALS phenotypes. Further studies are needed to replicate our findings in other cohorts and assess the relationship of protein alterations between peripheral blood and CSF.

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Author Contributions

JXZ and QQZ conceived and designed the study. JXZ, QQZ, QL, QN, WPG, DDS, SZL, BX, and FFB acquired, analyzed, and interpreted the data. JXZ, QQZ, and FFB drafted the manuscript, figures and tables. All authors contributed to the revision of the manuscript for important intellectual content.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Figure Captions

